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TITLE OF THE INVENTION (280 characters max)

IMMUNIZATION WITH *Porphyromonas gingivalis* PROTECTS AGAINST HEART DISEASE

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PROVISIONAL APPLICATION FILING ONLY

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IMMUNIZATION WITH *Porphyromonas gingivalis* PROTECTS AGAINST HEART DISEASE

Porphyromonas gingivalis, a gram negative, anaerobic bacterium is well accepted as the source of the majority of adult periodontal disease (PD) in humans. Currently there are no vaccines in clinical use for control of adult periodontal disease. Conflicting epidemiological data and case reports hint that adult PD may represent a significant risk factor for cardiovascular disease (CVD) in humans. Our recent studies demonstrate that hyperlipidemic mice, homozygous negative at the ApoE gene locus (ApoE$^{-/-}$), when orally challenged with wild type *P. gingivalis*, develop accelerated atherosclerotic plaque. Most important is our observation that immunization with our vaccine to prevent *P. gingivalis*-elicited periodontal disease prior to oral challenge concomitantly reduces atheroma formation in ApoE$^{-/-}$ mice orally challenged with *P. gingivalis* to levels observed in the unchallenged control ApoE$^{-/-}$ mice.

Thus, the invention is directed to the use of a vaccine against *P. gingivalis* to reduce the risk of CVD in humans that are at "high risk" for developing PD and CVD. We have shown that prophylactic immunization with such a vaccine prevents *P. gingivalis*-accelerated atheroma development in hyperlipidemic ApoE$^{-/-}$ mice. The tested vaccine was a preparation of heat-killed *P. gingivalis* suspended in phosphate buffered saline and consisted of all antigens present as the result of the heat-killing process. Adjuvant was not included in the vaccine and was not used during these studies. Our initial studies show that through immunization with our vaccine candidate to prevent *P. gingivalis* oral infection and subsequent oral bone loss, we reduce the ability of *P. gingivalis* to accelerate atherosclerotic plaque formation. This vaccine provides a method of controling one of the significant risk factors for CVD, *P. gingivalis* oral infection. Several groups of patients could be helped significantly by this vaccine including: 1- patients with a familial history of both PD and CVD, 2- patients with clinical signs of developing PD that already possess a know risk factor for CVD such as high cholesterol levels, and 3- other 'high risk" groups that have been shown to develop accelerated PD and CVD, such as diabetics. The vaccine in its current form has demonstrated prophylactic efficacy in a rodent model of accelerated atherosclerosis, as mice immunized prior to *P. gingivalis* oral challenge were protected from *P. gingivalis*-accelerated atheroma development. This supports prophylactic use of this vaccine. As it is unknown whether PD precedes or follows CVD, we envision that a vaccine against *P. gingivalis* may also possess therapeutic usefulness and provide the benefit of halting the effects of PD on the developing CVD condition.

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The Cardiovascular Disease Periodontal Disease Link: Common Theme of Control Through Immunization

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Abstract

Epidemiological studies support a clinical connection between periodontal disease, a chronic inflammatory disease of the supporting tissues of the teeth, and cardiovascular disease. To test this connection, hyperlipidemic (ApoE$^{-/-}$) mice were orally challenged with *Porphyromonas gingivalis*, the principal bacterium associated with adult periodontal disease, or an invasion-impaired *P. gingivalis* fimbriae-deficient mutant (FimA-). Challenge with wild type *P. gingivalis*, but not with the FimA- mutant, induced oral bone loss, expression of toll-like receptors-2, and -4, and accelerated athrerosclerotic plaque formation in ApoE$^{-/-}$ mice. Immunization of ApoE$^{-/-}$ mice to inhibit *P. gingivalis* oral infection concomitantly prevented *P. gingivalis*-accelerated atherosclerosis.

Body of Manuscript

Cardiovascular disease (CVD) is one of the primary causes of death in the western world (1), and despite identification and thorough study of several important factors that predispose humans to CVD including diet, metabolism, exercise, and genetics, an incomplete picture of the etiology of CVD is evident as these factors alone fail to completely predict cardiac events (2). A more thorough understanding of the mechanisms underlying the pathogenesis of CVD has emerged due in part to the use of gene-targeted animals such as the ApoE knockout ($ApoE^{-/-}$) mouse that develop severe hyperlipidemia, and accelerated atheroma formation (3). Recent investigations have established the importance of inflammation as a determining factor for development of CVD, with emphasis on a mononuclear cellular infiltrate (4), the host response to ox-LDL (5), cytokines (6), C-reactive protein levels (7), cell adhesion molecule expression (8), and identification of Toll-like receptor (TLR)-4 at the site of atherosclerotic plaque accumulation (9). **TLRs are an emerging group of pattern recognition molecules that mediate the innate host response to microbial infection (10), and recent reports demonstrate that TLRs are selectively up-regulated following infection, as part of the host innate response (11, 12).** Due to the reported parallels of several important CVD markers with the host response to infectious diseases, an infectious etiology component to CVD has been suggested (13). It has been reported that *Chlamydia*, *Helicobacter*, Herpes simplex virus, and Cytomeglovirus infection may be linked to CVD (14, 15). However, the connection between infectious diseases, and initiation and/or acceleration of CVD remains speculative due to conflicting reports (16).

Periodontal disease, a chronic inflammatory disease of the periodontium that leads to erosion of the attachment apparatus and supporting bone for the teeth (17), is one of the most common chronic infectious diseases of humans (18). *Porphyromonas gingivalis* is the primary etiologic agent of adult periodontal disease (19, 20). Epidemiological data, and case reports suggest that *P. gingivalis* oral infection represents a significant risk factor for CVD (21, 22); however, other reports do not confirm the clinical connection between periodontal disease, and increased risk for CVD (**JAMA REF**) (23). Haraszthy *et al.* (24) recently detected *P. gingivalis* in human atheromatous tissue by polymerase chain reaction, indicating that *P. gingivalis* present in the oral cavity gains access to the vasculature. These results suggest that bacteremia followed by subsequent attachment and invasion of the vascular endothelium by *P. gingivalis* may be responsible for localization of *P. gingivalis* to this site. Experimental studies further support the hypothesis that *P. gingivalis* can aggravate CVD as *P. gingivalis* bacteremia (25), systemic dissemination of *P. gingivalis* with extra-oral infection (26), and injection of *P. gingivalis* into blood vessels of mice stimulates atheroma formation (27). Additionally, *in vitro* studies have reported that *P. gingivalis* invades human endothelial cells (28), elicits cell adhesion molecules (29), and chemokines (30), while a *P. gingivalis* fimbriae-deficient mutant was significantly impaired in invasion, and failed to activate cells *in vitro* (28, 29, 30). The importance of fimbriae for *P. gingivalis*-elicited oral disease pathogenesis has been confirmed *in vivo*, as a *P. gingivalis* fimbriae-deficient mutant failed to elicit oral bone loss using a rat oral infection model (31).

Based on these observations, we hypothesized that oral infection of hyperlipidemic ApoE$^{-/-}$ mice with *P. gingivalis*, in a manner that elicits oral bone loss

(32), would stimulate accelerated atherosclerotic plaque accumulation. Pilot experiments were initially performed to determine if oral infection of hyperlipidemic $ApoE^{-/-}$ mice with wild type *P. gingivalis* strain 381 leads to dissemination of *P. gingivalis* (escape from the oral environment), as well as detection of *P. gingivalis* in the aortic tissues (localization of *P. gingivalis* in the tissues associated with accelerated atheroma formation). Groups of C57BL-6, and $ApoE^{-/-}$ mice, placed on a normal chow diet, were challenged orally (32) over a 3-week period (n=15 challenges) with either wild type *P. gingivalis* strain 381, or an insertional mutant deficient in major fimbriae *P. gingivalis* strain DPG3 (FimA-). This mutant is impaired in attachment, and invasion of epithelial, and endothelial cells *in vitro* (28), fails to elicit inflammatory markers from these cells *in vitro* (29, 30), and is not a potent stimulator of oral bone loss (31). We included groups of unchallenged C57BL-6, and $ApoE^{-/-}$ mice to serve as age-matched control populations. Blood samples were obtained from $ApoE^{-/-}$ mice 2 h after the final oral challenge with wild type or mutant *P. gingivalis*, and the aortic arches were obtained 24 h after the final oral challenge. Both wild type *P. gingivalis* and the FimA- mutant were detected in the blood of $ApoE^{-/-}$ mice by PCR amplification of the *P. gingivalis* 16S rRNA gene (Fig. 1A) (33, 34). Additionally, PCR amplification of *P. gingivalis* 16S gene from $ApoE^{-/-}$ mouse aortic arch tissue revealed that both wild type, and mutant *P. gingivalis* were present in these tissues (Fig. 1B).

To directly test the hypothesis that *P. gingivalis*-elicited periodontal disease accelerates atherosclerotic plaque accumulation, groups of 6-week-old, male, C57BL-6 and $ApoE^{-/-}$ mice were placed on a normal chow diet, and were challenged orally with either wild type *P. gingivalis*, or the *P. gingivalis* FimA- mutant, as described above. All

animals were sacrificed 6-weeks after the final *P. gingivalis* oral challenge, and were 17-weeks of age (35). As anticipated, C57BL-6 mice, either unchallenged or orally challenged with either strain of *P. gingivalis*, possessed low cholesterol and triglyceride levels (data not shown). Unchallenged ApoE$^{-/-}$ mice possessed high cholesterol and triglyceride levels, and oral challenge with either the wild type or the *P. gingivalis* FimA- mutant had no effect on the serum levels of these molecules, demonstrating that *P. gingivalis* oral infection does not lead to modified serum lipid levels in the host (Fig. 2A). Analysis of sera for total *P. gingivalis*-specific IgG revealed no significant differences in the adaptive host response of ApoE$^{-/-}$ mice following challenge with wild type *P. gingivalis*, or the FimA- mutant (Fig. 2B). No differences were observed in the ability of wild type *P. gingivalis* to elicit oral bone loss in C57BL/6 or ApoE$^{-/-}$ mice (data not shown). Additionally, we observed that both C57BL/6, and ApoE$^{-/-}$ mice orally challenged with the *P. gingivalis* FimA- mutant did not exhibit significant bone loss (data not shown), and this later observation is in agreement with previous studies reported in a rat model (31).

To determine if *P. gingivalis* oral infection leads to accelerated atheroma formation, the aorta of each mouse was harvested from the aortic valve to the iliac bifurcation and morphometric, and immunohistochemical analyses were performed (27, 36). It is well established that the accumulation of atherosclerotic plaque on the intimal surface of ApoE$^{-/-}$ mouse aortas occurs at the aortic valves and continues into the aortic arch area (15, 37, 38, 39). Based on this, we determined the total amount of atherosclerotic plaque present on the intimal surface from the aortic valves through the arch. *En face* measurements of the Sudan IV stained atherosclerotic plaque accumulation

revealed that $ApoE^{-/-}$ mice challenged orally with wild type *P. gingivalis* possessed significantly more atherosclerotic plaque accumulation on the intimal surface of the aortic arch as compared to unchallenged $ApoE^{-/-}$ mice (Fig. 2C and Fig. 2D). However, despite detection of the *P. gingivalis* mutant in the blood 2 h after oral challenge, and in aortic arch tissue 24 h after oral challenge, $ApoE^{-/-}$ mice challenged orally with the FimA-mutant failed to accelerate atheroma development, as the level of deposited atherosclerotic plaque resembled unchallenged animals (Fig. 2E and Fig. 2F)(40). We conclude that only fully invasive *P. gingivalis* initiate accelerated plaque accumulation (Fig. 2F), and infer that neither localization of *P. gingivalis* due to capture by the spontaneously developing plaque, nor the presence of *P. gingivalis* as demonstrated by PCR is sufficient to drive accelerated atheroma formation. The observed atherosclerotic plaque accumulation elicited by wild type *P. gingivalis* oral challenge did not progress to the thoracic, or abdominal regions of the aorta of $ApoE^{-/-}$ mice. We believe that the lack of progression of the plaque into these regions of the aorta may be a function of the chow diet, and age of these animals (17-weeks of age) (39, 41).

Since only the wild type *P. gingivalis* phenotype stimulated accelerated atheroma formation, and we did not observe differences in the adaptive immune response to wild type *P. gingivalis* or the mutant, we hypothesized that differences in the innate immune response to *P. gingivalis* plays a role in *P. gingivalis*-accelerated atheroma formation. **Recently, it was reported that the pattern recognition receptor TLR-4, a marker of the innate immune response (13), is up regulated in human, and mouse atheromatous vascular tissues (9).** RT-PCR amplification for TLR-2, and TLR-4 in aortic tissue of *P. gingivalis* orally challenged $ApoE^{-/-}$ mice revealed increased expression

of TLR-2 and TLR-4 gene transcription in the mice orally challenged with wild type *P. gingivalis*. The *P. gingivalis* FimA- mutant challenged mice were negative for TLR-2 and TLR-4, and resembled unchallenged animals (Fig. 3A). **TLR-2 and TLR-4 expression in the aortas of orally challenged ApoE$^{-/-}$ mice was confirmed by immunohistochemistry (9, 42). TLR-2 was detected in aortic tissue of all ApoE$^{-/-}$ mice and was localized primarily at athereromatous lesions (Fig. 3B). Low levels of TLR-2 were observed in aortic tissues of unchallenged mice. Elevated TLR-2 levels were observed in aortic tissue sections from ApoE$^{-/-}$ mice orally challenged with wild type *P. gingivalis*. Slight TLR-2-specific staining was observed in tissue sections from mutant *P. gingivalis* challenged mice. TLR-4 was observed only in the aortic sinus of ApoE$^{-/-}$ mice orally challenged with invasive *P. gingivalis* (Fig. 3B). While aortic tissue from ApoE$^{-/-}$ mice challenged with the *P. gingivalis* FimA- mutant failed to express TLR-4, and resembled unchallenged mice (Fig. 3B).** To confirm this observation, we infected primary explant cultures of human aortic endothelial cells to determine if *P. gingivalis* is able to activate TLR expression on aortic endothelial cells *in vitro*. We observed that human aortic endothelial cells infected with wild type *P. gingivalis* at a MOI of 100 elicited surface expressed TLR-2, and TLR-4 on these cells by FACS, while the FimA- mutant failed to stimulate TLR-2, or TLR-4 expression, and resembled control cells **(data not shown; ???SUPPL DATA???). Unexpectedly, we observed that human aortic endothelial cells cultured with the purified FimA protein did not lead to up-regulated expression of either TLR-2 or TLR-4 (data not shown; ???SUPPL DATA???).** These *in vitro* and *in vivo* data support that oral challenge of ApoE$^{-/-}$ mice with *P. gingivalis* leads to a host innate immune response to

the fully invasive *P. gingivalis*, and fails to initiate an innate host response (as defined by TLR-regulation) in the aortic tissue to the *P. gingivalis* FimA- mutant. Taken together these data indicate that despite initial detection of *P. gingivalis* in the blood, and in the aortic tissue, only wild type *P. gingivalis* infection stimulates an innate immune response as defined by differential TLR expression. This suggests that merely the presence of *P. gingivalis* or *P. gingivalis* antigens is not sufficient to initiate and drive accelerated atheroma development as only wild type *P. gingivalis* elicited accelerated atherosclerotic plaque accumulation. Additionally, these data suggest that differences in the innate host response to wild type *P. gingivalis*, as it directly relates to the mechanism of invasion, correlates with the clinical outcome of atheroma formation.

Based on our observations that oral challenge of ApoE$^{-/-}$ mice with invasive *P. gingivalis* 1- elicited oral bone loss, and 2- accelerated atherosclerotic plaque accumulation, we hypothesized that immunization of ApoE$^{-/-}$ mice to prevent periodontal disease would ameliorate *P. gingivalis*-accelerated atheroma development. To date there are no vaccines in clinical use for prevention of periodontal disease; however, vaccine candidates including heat-killed *P. gingivalis* have been tested in murine models and demonstrate prophylactic control of *P. gingivalis*-elicited oral bone loss (32). To test the hypothesis that immunization to protect animals from oral bone loss, concomitantly prevents accelerated atherosclerosis, groups of ApoE$^{-/-}$ mice (n = 10 mice /group) were immunized twice weekly for 3-weeks with a heat-killed *P. gingivalis* 381 whole organism preparation, without adjuvant, prior to oral challenge with wild type *P. gingivalis*. Additionally we included groups of unchallenged, and wild type *P. gingivalis* orally challenged age-matched ApoE$^{-/-}$ mice. Our results confirmed previous data that

active immunization of mice with heat-killed *P. gingivalis* elicited a potent *P. gingivalis*-specific serum IgG response (data not shown), and that immunization with heat-killed *P. gingivalis* prevented *P. gingivalis*-elicited oral bone loss (data not shown) (32). The *en face* morphometric analysis of Sudan IV stained atherosclerotic plaque accumulation on the intimal surface of the aortic arch of ApoE$^{-/-}$ mice revealed that immunization with heat-killed *P. gingivalis* prior to oral challenge protected animals from *P. gingivalis*-accelerated atheroscleotic plaque accumulation (Fig. 4A, B, C, and D). These results demonstrate that by using an effective immunization strategy to prevent periodontal disease, we can completely prevent *P. gingivalis* accelerated atherosclerosis formation.

Although conflicting reports exist regarding the ability of infectious diseases to aggravate CVD, this study using wild type *P. gingivalis*, and a defined *P. gingivalis* fimbriae-deficient mutant clearly demonstrate that a specific mechanism by which this bacterial pathogen interacts with the host, and the subsequent host immune response to this pathogen in the aorta, are linked to accelerated atherosclerotic plaque deposition. Of particular interest, we report that immunization to prevent *P. gingivalis*-mediated periodontal disease, also provides the added benefit of significantly reducing the acceleration of CVD. As adult periodontal disease affects an estimated 30% of humans over the age of 35 (18), and cardiovascular disease in one of the major causes of death in the western world (1), further assessment of the interaction of oral pathogens with the host is necessary to develop adequate understanding of the complex processes that link these two significant human diseases.

In summary, although CVD is a multifactorial disease, using a combinational approach consisting of a defined genetic mutant of *P. gingivalis*, a site-specific challenge

regimen, and immunization studies performed within an effective animal model for assessment of accelerated atherosclerosis, we have demonstrated an experimental link between *P. gingivalis* oral infection and exacerbation of atherosclerotic plaque accumulation in ApoE$^{-/-}$ mice. Our data indicate that the mechanism by which *P. gingivalis* accelerates atherosclerosis likely requires bacterial attachment and invasion, as an attachment / invasion mutant failed to accelerate atheroma formation. These data demonstrate that there is a direct impact of the chronic oral disease periodontitis, on the development / acceleration of CVD. Most importantly, our data suggest that vaccination to prevent periodontal disease caused by *P. gingivalis* may concomitantly reduce the risk of accelerated CVD in high-risk groups.

Figure Legends

Fig. 1. Following oral challenge, *P. gingivalis* gains access to the circulatory system and localizes in the aortic arch of ApoE$^{-/-}$ mice. **(A)** PCR amplification of *P. gingivalis* 16S rRNA gene to detect wild type *P. gingivalis* strain 381 (WT), and mutant *P. gingivalis* (FimA-) from blood 2 h after the final oral challenge. **(B)** PCR detection of *P. gingivalis* in aortic tissue of ApoE$^{-/-}$ mice orally challenged with wild type (WT), or mutant (FimA-) *P. gingivalis* 24 h after oral challenge. Note that both blood and aortic arch tissue are positive for *P. gingivalis* by PCR amplification of the 16S gene. C = control *P. gingivalis* 381 DNA (527 bp product), M = 500 bp molecular weight standard, None = unchallenged ApoE$^{-/-}$ mouse.

Fig. 2. Oral challenge of ApoE$^{-/-}$ mice with wild type *P. gingivalis* stimulates accelerated atherosclerotic plaque formation 6-weeks following oral challenge. **(A)** Serum analysis of total cholesterol, and triglyceride levels from ApoE$^{-/-}$ mice (n = 10 mice/group) at the time of sacrifice (6-weeks following final oral challenge). No statistical differences were observed between ApoE$^{-/-}$ mouse serum levels of total cholesterol, or triglyceride levels in response to wild type *P. gingivalis* (gray bars), or the *P. gingivalis* FimA- mutant (diagonally hatched bars) challenge, and resembled unchallenged levels (black bars) using Student's two tailed t test. Data presented as the mean ± SD. **(B)** Assessment of the host adaptive immune response of ApoE$^{-/-}$ mice to *P. gingivalis*. Serum levels of *P. gingivalis*-specific IgG was determined by quantitative ELISA prior to oral challenge (blue bars), or at 6-weeks following oral challenge (purple bars). Both wild type *P. gingivalis* (WT), and the mutant *P. gingivalis* (FimA-) elicited similar levels of *P.*

gingivalis-specific IgG (P>0.9 by Student's two tailed t test). Data presented as the mean ± SD. Figures **C**, **D**, and **E** are representative of the atherosclerotic plaque present on the intimal surface of *en face* Sudan IV stained aortic arches of ApoE$^{-/-}$ mice. **(C)** Unchallenged mouse; **(D)** mouse orally challenged with the wild type *P. gingivalis*; and **(E)** mouse orally challenged with the *P. gingivalis* FimA- mutant. **(F)** Morphometric analysis of the total area of atherosclerotic plaque deposited in the aortic arch of ApoE$^{-/-}$ mice (n = 10 mice/group). ApoE$^{-/-}$ mice orally challenged with wild type *P. gingivalis* (WT) stimulated significantly more atherosclerotic plaque deposition as compared with unchallenged (None), or mutant *P. gingivalis* (FimA-) challenged animals (* = P<0.05 by Student's two tailed t test). The mutant failed to elicit accelerated atherosclerotic plaque deposition, and the accumulated plaque resembled unchallenged mice (NS = P > 0.7 by Student's two tailed t test). Data presented as the mean ± SD.

Fig. 3. ApoE$^{-/-}$ mice orally challenged with invasive *P. gingivalis* express increased TLR-2 and TLR-4 in aortic arch tissue 6-weeks following oral challenge. **(A)** RT-PCR amplification of TLR-2, and TLR-4 mRNA from aortic arch tissue of either unchallenged (None) ApoE$^{-/-}$ mice, or ApoE$^{-/-}$ mice orally challenged with either wild type (WT), or mutant *P. gingivalis* (FimA-). Unchallenged ApoE$^{-/-}$ mice expressed low levels of TLR-2 mRNA, and did not express TLR-4 mRNA. ApoE$^{-/-}$ mice orally challenged with wild type *P. gingivalis* expressed high levels of TLR-2, and TLR-4 mRNA, while mice challenged with the FimA- mutant did not express TLR-2, or TLR-4. **(B)** Immunohistochemical confirmation of TLR-2, and TLR-4 expression in aortic tissue. As expected, TLR-2 was localized primarily to the sites of atherosclerotic plaque in ApoE$^{-/-}$

mouse aortic arch tissues. Elevated levels of TLR-2, and TLR-4 protein (brown staining) were observed in aortic tissue of ApoE$^{-/-}$ mice orally challenged with wild type *P. gingivalis*. Tissue from the aortic arch of unchallenged ApoE$^{-/-}$ mice and *P. gingivalis* mutant orally challenged ApoE$^{-/-}$ mice expressed low levels of TLR-2, and failed to express TLR-4. Irrelevant isotype-matched antibody (IRR-Ab) probed sections were negative for TLR-2 or TLR-4. Original magnification of 100 x for all photomicrographs.

Fig. 4. Immunization of mice with heat-killed *P. gingivalis* prevents *P. gingivalis*-accelerated atherosclerotic plaque accumulation. Figures **A**, **B**, and **C** are representative of the intimal surface of Sudan IV stained atherosclerotic plaque in the aortic arch of ApoE$^{-/-}$ mice, 6-weeks following oral challenge; **(A)** unchallenged mouse; **(B)** animal orally challenged with the wild type *P. gingivalis*; **(C)** mouse immunized with heat-killed *P. gingivalis* without adjuvant, and orally challenge with wild type *P. gingivalis*. **(D)** Morphometric analysis of the total area of atherosclerotic plaque deposited on the intimal surface of the aortic arch of ApoE$^{-/-}$ mice (n = 10 mice/group). Mice orally challenged with wild type *P. gingivalis* (WT) possessed significant atherosclerotic plaque as compared to unchallenged (None) mice (* = P<0.05 by Student's two tailed t test). Immunized mice (Immun + WT) were protected from wild type *P. gingivalis*-elicited accelerated atherosclerotic plaque accumulation (** = P<0.05 vs. ApoE$^{-/-}$ mice orally challenged with wild type *P. gingivalis* by Student's two tailed t test), and resembled unchallenged (None) mice (NS = P>0.7 by Student's two tailed t test). Data presented as the mean ± SD.

Figure S1: Infection of human aortic endothelial cells with wild type *P. gingivalis* stimulates TLR-2 and TLR-4 expression. FACS analysis of TLR-2 and TLR-4 expression on human aortic endothelial cells (Cascade Biologics, Portland, Oregon) cultured with wild type *P. gingivalis* (red trace), or the FimA- mutant (blue trace) at an MOI of 100, and unstimulated cells functioned as controls (shaded black trace). TLR-2 and TLR-4 expression was up-regulated on aortic endothelial cells at both 2 and 6 h post challenge. By 24 h TLR expression was not evident and resembled unchallenged TLR expression levels.

Figure S2: Human aortic endothelial cells cultured with the purified *P. gingivalis* FimA protein do not express TLR-2 or TLR-4. FACS analysis of TLR-2 and TLR-4 expression on human aortic endothelial cells (Cascade Biologics, Portland, Oregon) cultured with either high dose of *P. gingivalis* FimA (10 μg/ml; red trace), low dose *P. gingivalis* FimA (1 μg/ml; blue trace), or were unstimulated (black trace). As a positive control stimulus for TLR-2 and TLR-4 expression, cells were stimulated with wild type *P. gingivalis* at a MOI of 100 (shaded trace). TLR-2 and TLR-4 expression was not evident on aortic endothelial in response to either high or low concentration of the FimA protein, and resembled unchallenged cells, at all time points tested.

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References and Notes

1. R. Ross, *Nature* **362**, 801-9. (1993).
2. P. Libby, *Am J Cardiol* **88**, 3J-6J. (2001).
3. S. H. Zhang, R. L. Reddick, J. A. Piedrahita, N. Maeda, *Science* **258**, 468-71. (1992).
4. F. Mach, et al., *J Clin Invest* **104**, 1041-50. (1999).
5. D. Steinberg, *J Biol Chem* **272**, 20963-6. (1997).
6. S. Gupta, et al., *J Clin Invest* **99**, 2752-61. (1997).
7. G. Liuzzo, et al., *N Engl J Med* **331**, 417-24. (1994).
8. M. I. Cybulsky, et al., *J Clin Invest* **107**, 1255-62. (2001).
9. X. H. Xu, et al., *Circulation* **104**, 3103-8. (2001).
10. E. Lien, R. R. Ingalls, *Crit Care Med* **30**, S1-S11. (2002).

11. T. Shuto, et al., *J Biol Chem* **277**, 17263-70. (2002).

12. T. Wang, W. P. Lafuse, B. S. Zwilling, *J Immunol* **165**, 6308-13. (2000).

13. G. K. Hansson, P. Libby, U. Schonbeck, Z. Q. Yan, *Circ Res* **91**, 281-91. (2002).

14. S. E. Epstein, Y. F. Zhou, J. Zhu, *Circulation* **100**, e20-8. (1999).

15. F. Mach, G. K. Sukhova, M. Michetti, P. Libby, P. Michetti, *Circ Res* **90**, E1-4. (2002).

16. S. D. Wright, et al., *J Exp Med* **191**, 1437-42. (2000).

17. G. C. Armitage, *Ann Periodontol* **1**, 37-215. (1996).

18. R. C. Oliver, L. J. Brown, H. Loe, *J Periodontol* **69**, 269-78. (1998).

19. S. C. Holt, J. Ebersole, J. Felton, M. Brunsvold, K. S. Kornman, *Science* **239**, 55-7. (1988).

20. A. L. Griffen, M. R. Becker, S. R. Lyons, M. L. Moeschberger, E. J. Leys, *J Clin Microbiol* **36**, 3239-42. (1998).

21. J. D. Beck, et al., *Arterioscler Thromb Vasc Biol* **21**, 1816-22. (2001).

22. T. Wu, et al., *Arch Intern Med* **160**, 2749-55. (2000).

23. J. D. Beck, S. Offenbacher, R. Williams, P. Gibbs, R. Garcia, *Ann Periodontol* **3**, 127-41. (1998).

24. V. I. Haraszthy, J. J. Zambon, M. Trevisan, M. Zeid, R. J. Genco, *J Periodontol* **71**, 1554-60. (2000).

25. M. Messini, et al., *J Clin Periodontol* **26**, 469-73. (1999).

26. S. C. Lee, C. P. Fung, C. C. Lin, C. J. Tsai, K. S. Chen, *J Microbiol Immunol Infect* **32**, 213-6. (1999).

27. L. Li, E. Messas, E. L. Batista, Jr., R. A. Levine, S. Amar, *Circulation* **105**, 861-7. (2002).

28. R. G. Deshpande, M. Khan, C. A. Genco, *Invasion Metastasis* **18**, 57-69 (1998).

29. M. Khlgatian, H. Nassar, H. H. Chou, F. C. Gibson, 3rd, C. A. Genco, *Infect Immun* **70**, 257-67. (2002).

30. H. Nassar, et al., *Infect Immun* **70**, 268-76. (2002).

31. R. Malek, et al., *J Bacteriol* **176**, 1052-9. (1994).

32. F. C. Gibson, 3rd, C. A. Genco, *Infect Immun* **69**, 7959-63. (2001).

33. L. Garcia, et al., *J Periodontal Res* **33**, 59-64. (1998).

34. A 10 µl sample of whole blood was plated directly on to blood agar plates and cultivated anaerobically for up to 10 days. Additionally, 100 µl of whole blood from each mouse was used to detect the presence of the *P. gingivalis* 16S gene by PCR amplification (33).. As transient *P. gingivalis* bacteremia are well documented (25), we anticipated that blood cultures obtained from ApoE$^{-/-}$ mice challenged with wild type *P. gingivalis* would be positive. Unexpectedly, all blood cultures were negative for bacterial growth.

35. T. C. Moazed, L. A. Campbell, M. E. Rosenfeld, J. T. Grayston, C. C. Kuo, *J Infect Dis* **180**, 238-41. (1999).

36. P. K. Shah, et al., *Circulation* **97**, 780-5. (1998).

37. J. H. Qiao, et al., *Arterioscler Thromb* **14**, 1480-97. (1994).

38. R. K. Tangirala, E. M. Rubin, W. Palinski, *J Lipid Res* **36**, 2320-8. (1995).

39. Y. Nakashima, A. S. Plump, E. W. Raines, J. L. Breslow, R. Ross, *Arterioscler Thromb* **14**, 133-40. (1994).

40. C57BL-6 mice orally challenged with wild type or mutant *P. gingivalis* failed to develop atherosclerotic plaque accumulation on the intimal surface of the aortic arch, the thoracic, or abdominal regions of the aorta, or at any of the furcations, as these mice are resistant to atherosclerotic plaque accumulation when on a normal chow diet.

41. A. H. Lichtman, et al., *Arterioscler Thromb Vasc Biol* 19, 1938-44. (1999).

42. The TLR-2 monoclonal antibody was generated by immunizing Lewis rats with CHO/mouse TLR-2 cells and fusing the spleenocytes to NSO/1 mouse myeloma cells. A rat IgG2b, k antibody clone was chosen based on recognition of CHO/mo TLR-2, and non--reactivity with CHO/hu TLR-2 or CHO/hu TLR-4 by FACS. Nilsen, N., Nonstad, U., Sundan, A., Espevik, T. and Lien, E, manuscript in preparation.

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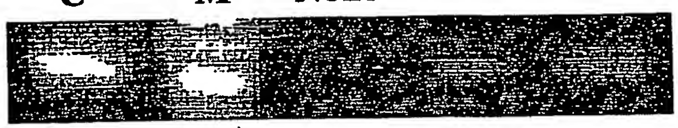
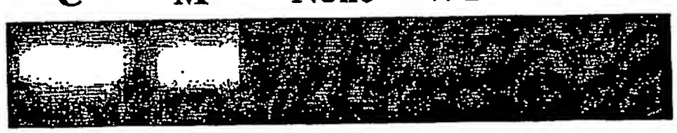
FIGURE 1

A



B



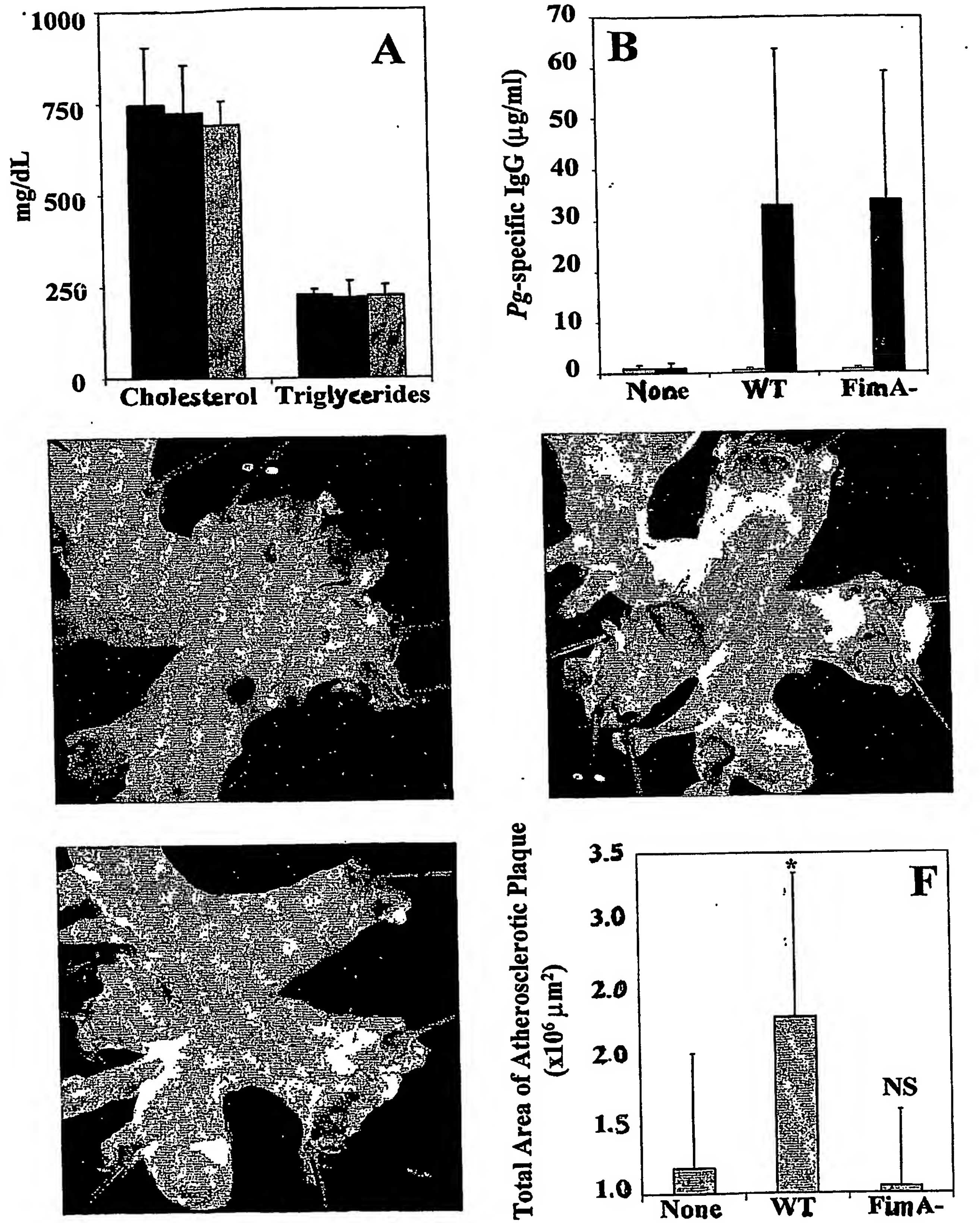

A
1000
750
500
250
0
mg/dL
Cholesterol
Triglycerides
B
70
60
50
40
30
20
10
0
Pg-specific IgG (µg/ml)
None
WT
FimA-
F
3.5
3.0
2.0
2.0
1.5
1.0
Total Area of Atherosclerotic Plaque
(x10⁶ µm²)
*
NS
None
WT
FimA-

FIGURE 3

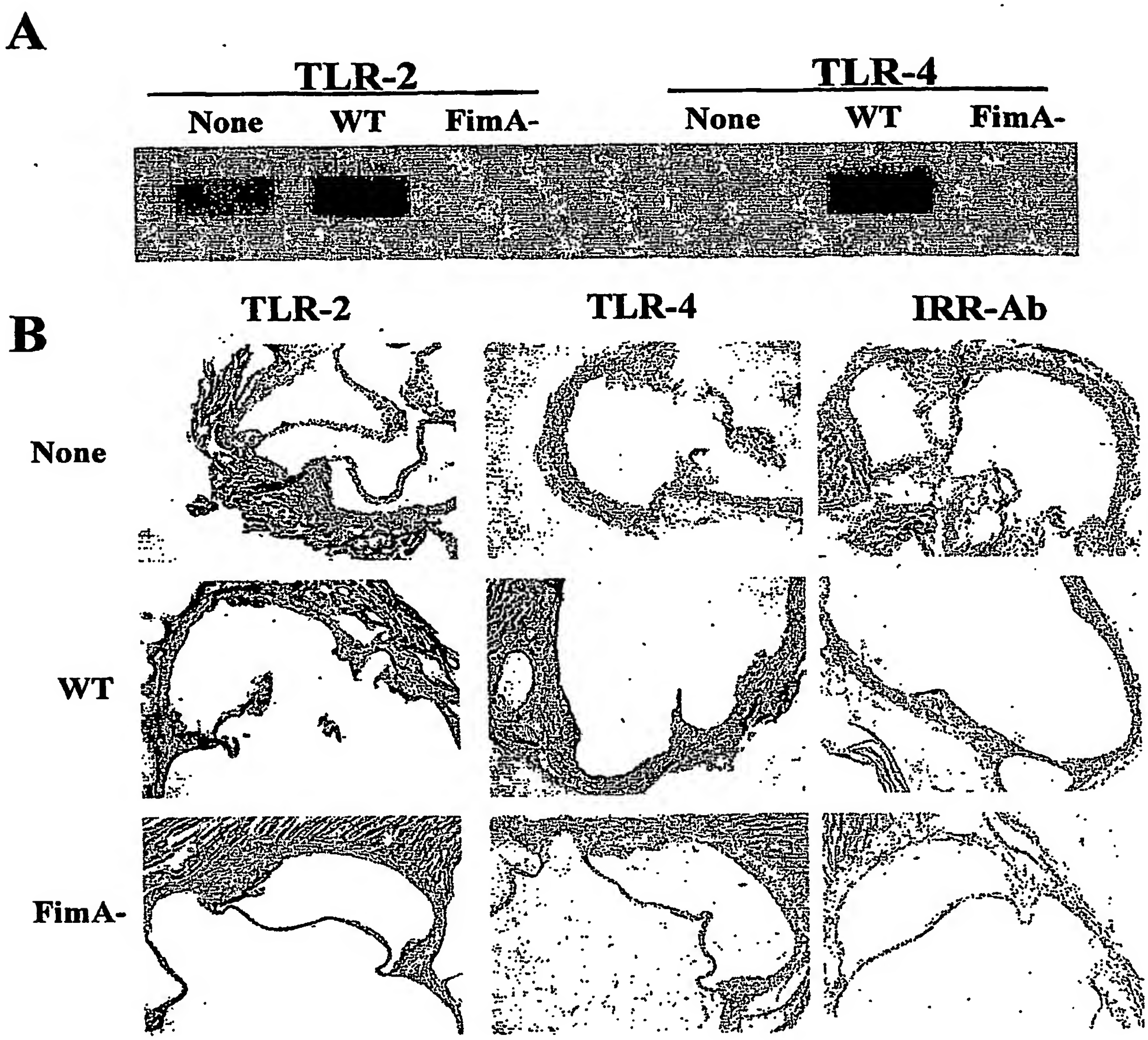
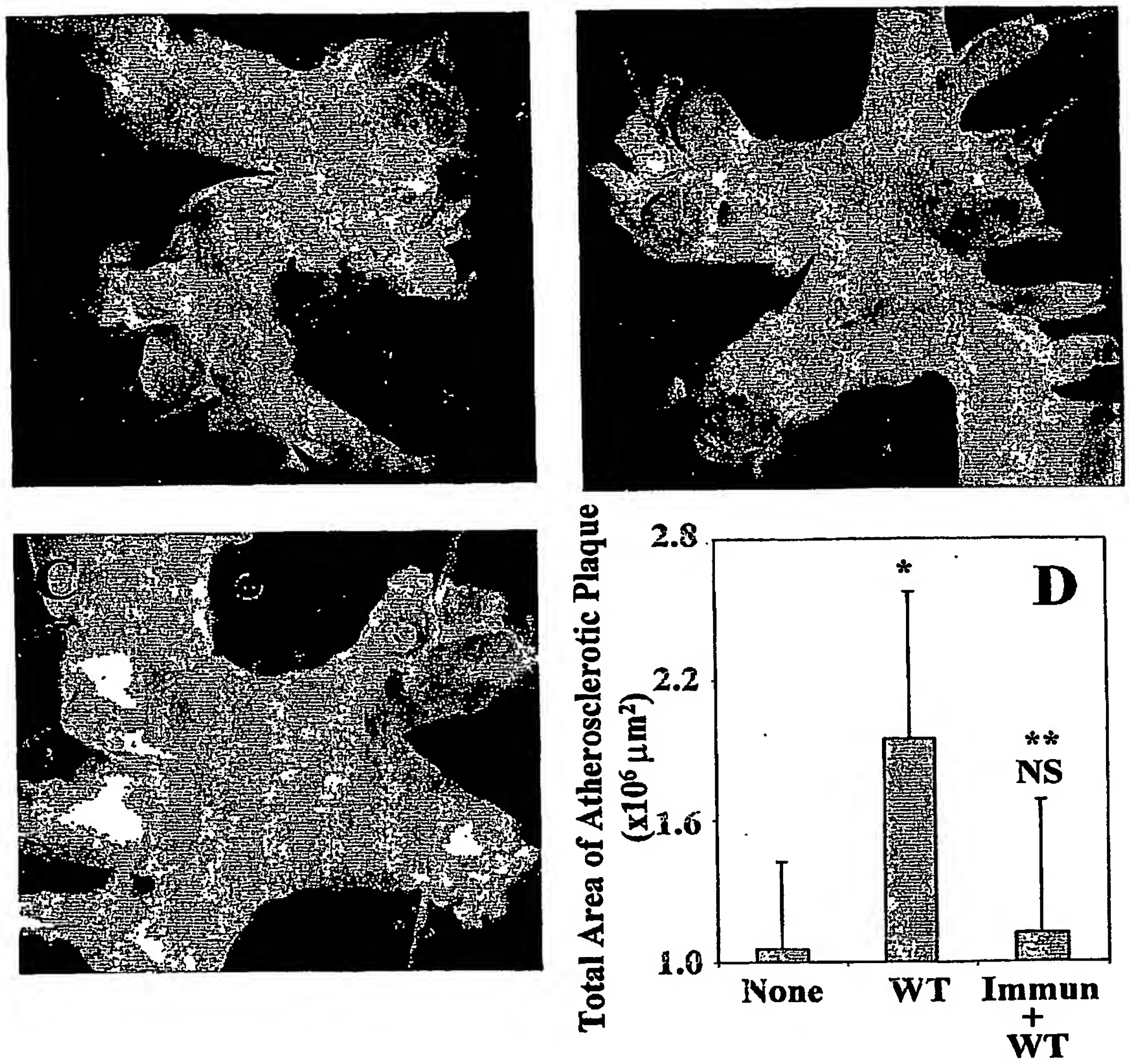
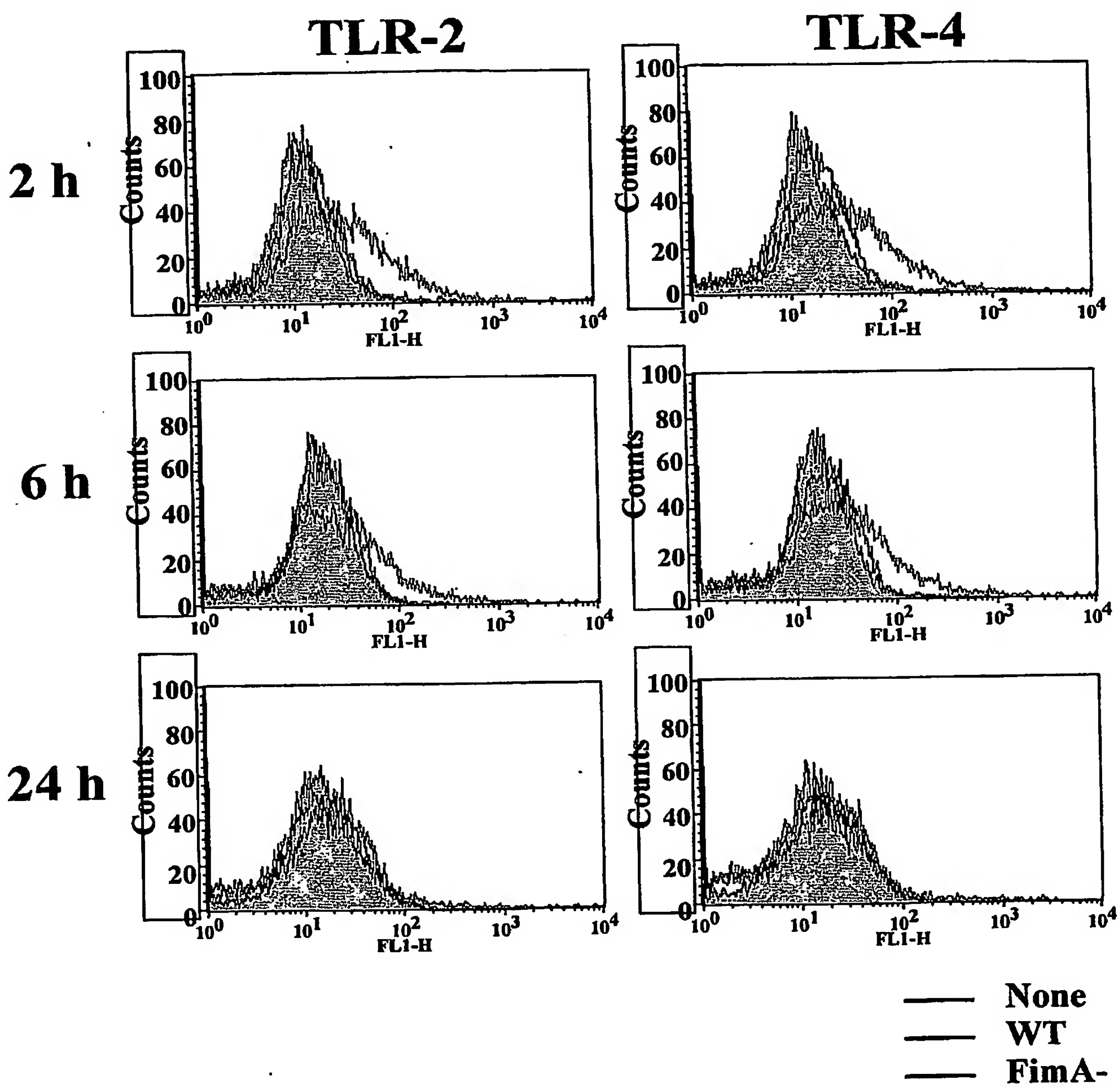

A
TLR-2
TLR-4
None
WT
FimA-
None
WT
FimA-
B
TLR-2
TLR-4
IRR-Ab
None
WT
FimA-

Figure 4


2.8
2.2
1.6
1.0
Total Area of Atherosclerotic Plaque
(x10⁶ μm²)
D
*
**
NS
None
WT
Immun
+
WT

Supplemental Data Figure 1
HAEC cells cultured with *P. gingivalis* Elicit TLR-2 and TLR-4



Supplemental Data Figure 2
HAEC cells cultured with *P. gingivalis* Fimbriae do not Elicit TLR-2 and TLR-4